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# PREVENTION OF DISULFIDE BOND REDUCTION DURING RECOMBINANT PRODUCTION OF POLYPEPTIDES

## CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 12/217,745, filed Jul. 8, 2008, which is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional Application No. 60/948,677 filed Jul. 9, 2007, the contents of which are incorporated herein by reference.

## FIELD OF THE INVENTION

The invention concerns methods and means for preventing the reduction of disulfide bonds during the recombinant production of disulfide-containing polypeptides. In particular, the invention concerns the prevention of disulfide bond reduction during harvesting of disulfide-containing polypeptides, including antibodies, from recombinant host cell cultures.

## BACKGROUND OF THE INVENTION

In the biotechnology industry, pharmaceutical applications require a variety of proteins produced using recombinant DNA techniques. Generally, recombinant proteins are produced by cell culture, using either eukaryotic cells, such as mammalian cells, or prokaryotic cells, such as bacterial cells, engineered to produce the protein of interest by insertion of a recombinant plasmid containing the nucleic acid encoding the desired protein. For a protein to remain biologically active, the conformation of the protein, including its tertiary structure, must be maintained during its purification and isolation, and the protein's multiple functional groups must be protected from degradation.

Mammalian cells have become the dominant system for the production of mammalian proteins for clinical applications, primarily due to their ability to produce properly folded and assembled heterologous proteins, and their capacity for post-translational modifications. Chinese hamster ovary (CHO) cells, and cell lines obtained from various other mammalian sources, such as, for example, mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293) and human retinal cells, such as the PER.C6® cell line isolated from a human retinal cell, which provides human glycosylation characteristics, and is able to naturally produce antibodies that match human physiology, have been approved by regulatory agencies for the production of biopharmaceutical products.

Usually, to begin the production cycle, a small number of transformed recombinant host cells are allowed to grow in culture for several days (see, e.g., FIG. 23). Once the cells have undergone several rounds of replication, they are transferred to a larger container where they are prepared to undergo fermentation. The media in which the cells are grown and the levels of oxygen, nitrogen and carbon dioxide that exist during the production cycle may have a significant impact on the production process. Growth parameters are determined specifically for each cell line and these parameters are measured frequently to assure optimal growth and production conditions.

When the cells grow to sufficient numbers, they are transferred to large-scale production tanks and grown for a longer period of time. At this point in the process, the recombinant protein can be harvested. Typically, the cells are engineered to

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secrete the polypeptide into the cell culture media, so the first step in the purification process is to separate the cells from the media. Typically, harvesting includes centrifugation and filtration to produce a Harvested Cell Culture Fluid (HCCF). The media is then subjected to several additional purification steps that remove any cellular debris, unwanted proteins, salts, minerals or other undesirable elements. At the end of the purification process, the recombinant protein is highly pure and is suitable for human therapeutic use.

Although this process has been the subject of much study and improvements over the past several decades, the production of recombinant proteins is still not without difficulties. Thus, for example, during the recombinant production of polypeptides comprising disulfide bonds, especially multi-chain polypeptides comprising inter-chain disulfide bonds such as antibodies, it is essential to protect and retain the disulfide bonds throughout the manufacturing, recovery and purification process, in order to produce properly folded polypeptides with the requisite biological activity.

## SUMMARY OF THE INVENTION

The instant invention generally relates to a method for preventing reduction of a disulfide bond in a polypeptide expressed in a recombinant host cell, comprising supplementing the pre-harvest or harvested culture fluid of the recombinant host cell with an inhibitor of thioredoxin or a thioredoxin-like protein.

In one embodiment, the thioredoxin inhibitor is added to the pre-harvest culture fluid.

In another embodiment, the thioredoxin inhibitor is added to the harvested culture fluid.

In a further embodiment, the thioredoxin inhibitor is a direct inhibitor of thioredoxin.

In all embodiments, the thioredoxin inhibitor may, for example, be an alkyl-2-imidazolyl disulfide or a naphthoquinone spiroketal derivative.

In a further embodiment, the thioredoxin inhibitor is a specific inhibitor of thioredoxin reductase.

In a still further embodiment, the thioredoxin inhibitor is a gold complex, where the gold complex may, for example, be aurothioglucose (ATG) or aurothiomalate (ATM). While the effective inhibitory concentration may vary, it typically is between about 0.1 mM and 1 mM. Similarly, the minimum effective inhibitory concentration varies depending on the nature of the polypeptide and overall circumstances, and is typically reached when the ATG or ATM concentration is at least about four-times of thioredoxin concentration in the pre-harvest or harvested culture fluid.

In another embodiment of this aspect of the invention, the thioredoxin inhibitor is a metal ion, where the metal ion, without limitation, may be selected from the group consisting of  $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Co}^{2+}$ , and  $\text{Mn}^{2+}$ . When the metal ion is added in the form of cupric sulfate, the effective inhibitory concentration generally is between about 5  $\mu\text{M}$  and about 100  $\mu\text{M}$ , or between about 10  $\mu\text{M}$  and about 80  $\mu\text{M}$ , or between about 15  $\mu\text{M}$  and about 50  $\mu\text{M}$ . The minimum inhibitory concentration of cupric sulfate also varies, but typically is reached when cupric sulfate is added at a concentration at least about two-times of thioredoxin concentration in the pre-harvest or harvested culture fluid.

In different embodiment, the thioredoxin inhibitor is an oxidizing agent, e.g., an inhibitor of G6PD, such as, for example, pyridoxal 5'-phosphate, 1 fluoro-2,4 dinitrobenzene, dehydroepiandrosterone (DHEA) or epiandrosterone (EA); cystine or cysteine. Typical effective inhibitor concen-